COMETABOLIC DEGRADATION OF CHLOROALLYL ALCOHOLS IN BATCH AND CONTINUOUS CULTURES

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Published in:
Applied Microbiology and Biotechnology

DOI:
10.1007/bf00170240

Appl Microbiol Biotechnol (1994) 42:158–166

ORIGINAL PAPER

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Received: 31 January 1994 / Received revision: 25 April 1994 / Accepted: 3 May 1994

Abstract The biodegradation of chloroallyl alcohols by pure and mixed bacterial cultures was investigated. Only 2-chloroallyl alcohol and *cis*– and *trans*–3-chloroallyl alcohol served as growth substrate for pure cultures. The other chloroallyl alcohols could be cometabolically degraded during growth on 2-chloroallyl alcohol. Cometabolic degradation of trichloroallyl alcohol, which was the most recalcitrant congener, by a *Pseudomonas* strain isolated on 2-chloroallyl alcohol resulted in 60% dechlorination. Efficient degradation of a mixture of chloroallyl alcohols in continuous culture could only be achieved in the presence of a satellite population. The mixed culture degraded 99% of the total chloroallyl alcohols added with 71% chloride release. The culture contained strains with a new catabolic potential. The results indicate the importance of mixed cultures and genetic adaptation for efficient chloroallyl alcohol removal.

Introduction

Biological treatment of industrial waste water has proven to be an efficient and reliable method for the removal of organic pollutants. Apart from the removal of lumped parameters such as biochemical oxygen demand and chemical oxygen demand, stricter water quality guidelines have prompted the development of biological treatment systems dedicated to the removal of individual components. The degradation of chlorinated xenobiotic compounds by pure and mixed cultures of bacteria has been the subject of intensive research in several laboratories. Most of this work was done with pure cultures. The degradation of mixed substrates by mixed cultures has received considerably less attention, but is certainly important for some compounds (Bitzi et al. 1991; Thurnheer et al. 1988). Cometabolic conversions may also play an important role in the degradation of more recalcitrant compounds, and may be of crucial importance for the efficient sanitation of complex waste streams. Chlorinated ethenes such as trichloroethylene and dichloroethylene are not known to support microbial growth, but they can be degraded by bacteria through the action of non-specific oxygenases (Ensley 1991). Cometabolic degradation of polychlorinated biphenyls is stimulated in the presence of biphenyl as the growth substrate (Kohler et al. 1988).

A hardly studied class of unsaturated chlorinated aliphatic compounds is formed by the chloroallyl alcohols, which are intermediates or byproducts in the synthesis of industrial herbicides (Rosen et al. 1980). 2-Chloroacrolein (2-chloropropenal) is found in waste water from paper mills that use chlorine bleaching, and is mutagenic (Motosugi and Soda 1983; Tachibana et al. 1989). Toxicity of other chloroallyl alcohols has also been attributed to the formation of the corresponding 2-haloacroleins (Meier et al. 1985; Rosen et al. 1980; Tachibana et al. 1989). The degradation of 2-chloroallyl alcohol by *Pseudomonas fluorescens* JD2 proceeds via oxidation of the alcohol to the corresponding acrylic acid, followed by dehalogenation (van der Waarde et al. 1993). Very little is known about the biodegradation of other chloroallyl alcohols. Metabolic biodegradation of the higher chlorinated allyl alcohols has not been described. We have attempted to isolate cultures that can grow on or mediate cometabolic degradation of these compounds. In addition, biological treatment of artificial waste water containing a mixture of chloroallyl alcohols was investigated in order to determine the feasibility of a biological treatment system.

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Materials and methods

Organisms and growth conditions

The following organisms were used in chloroallyl alcohol degradation experiments: the methanotrophs *Methylosinus trichosporium* OB3b (NCIB 11131) and *Methylosinus methanica* (NCIB 11130); the dichloromethane degrader *Hyphomicrobium* GJ21, isolated in our laboratory; the 1,2-dichloroethane degrader *Xanthobacter autotrophicus* GJ10 (Janssen et al. 1984); and several chloroallyl alcohol-utilizing *Pseudomonas* strains (van der Waarde et al. 1993; this report). Strain FG41 is a gram-positive allyl-chloride-utilizing-organism isolated by F. Fidom in our laboratory.

Cells were grown aerobically at 30° C under rotary shaking. To prevent evaporation of substrates, cultivation was carried out in flasks closed with Teflon-lined caps and filled to one-third of their volume with medium. The mineral medium (MMY) used in all experiments contained (l^{-1}) 5.3 g $Na_2HPO_4 \cdot 12H_2O$, 1.4 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 30 mg yeast extract (Difco Laboratories, Detroit, Mich.) and 5 ml salts solution (Janssen et al. 1984). Carbon sources were added at 5 mM, unless indicated otherwise. Acids were neutralized with KOH.

Methanotrophic bacteria were grown on MMF medium as described previously (Oldenhuis et al. 1989). In the case of *M. trichosporium* OB3b, copper sulfate was omitted from the trace-metal solution to allow derepression of the soluble methane monooxygenase. Natural gas was added to the gas phase (15%, v/v).

For continuous cultivation, cells were grown in fermentors with a working volume of approximately 600 ml. The temperature was kept constant at 30 ± 2° C. The pH was monitored and maintained at 7.0 ± 0.2 by automatic addition of 0.5 M NaOH or H_2SO_4. The cultures were sparged with water-saturated and filtered air (6 l/h) and stirred at 400 rpm. The mineral salts medium was the same as that for batch cultures, but with the concentration of phosphate buffer reduced to 1 mM. Where indicated, a cell recycle was used. Effluent from the fermentor entered the 0.3-l settler, and sedimented cells were automatically pumped back into the fermentor at regular intervals.

Cometabolism

Batch cultures containing a particular growth substrate at 2.5 mM and a cosubstrate where indicated (or a mixture of cosubstrates) were inoculated (25%, v/v) with a preculture grown on methane (5%, v/v in the gas phase) or on 5 mM growth substrate, and incubated at 30° C. At different times, samples were analyzed for chloride levels and substrate concentrations. Values were compared to those obtained in a batch culture with only 2.5 mM growth substrate added and a sterile control.

Analytical methods

Chlorinated allyl alcohols were quantitatively determined by capillary gas chromatography (GC), using a flame ionization detector. Samples of 4.5 ml were extracted with 1.5 ml ether, containing 0.05 mM 1-bromohexane as an internal standard. Extracts of samples were analyzed as described previously (van der Waarde et al. 1993).

Carboxylic acids were esterified with methanol. Samples (0.9 ml) were centrifuged (5 min, 120000 *g*) and added to 2 ml methanol and 0.2 ml 25% H_2SO_4. 3-Chloropropionic acid (10 mM) was added as an internal standard. The mixture was incubated at 100° C for 90 min and, after cooling, extracted with 2 ml hexane. The hexane layer was analyzed by gas chromatography on a CPWax-52CB column (Chrompack, Middelburg, The Netherlands) as described above.

Chloride levels were determined by the colorimetric method of Bergmann and Sanik (1957).

Chemicals

Chloroallyl alcohols were a kind gift from Dr. A. Aarts of Monsanto, Europe S.A., Louvain La Neuve, Belgium. When necessary, the compounds were purified by vacuum distillation until a purity of at least 95% was reached. 2,3-Dichloroallyl alcohol was only available as a mixture of the *cis*– and *trans*–isomers. All other organic compounds were obtained from commercial sources, and checked for purity by gas chromatography.

Results

Screening biodegradation of chloroallyl alcohols

Initial screening of the biodegradability of chloroallyl alcohols was done by incubating low concentrations of a specific chloroallyl alcohol with a suspension of soil or activated sludge. Both types of inocula came from industrial sites that had been exposed to chloroallyl alcohols, and possibly contained an adapted microflora. After 10 days of incubation, the concentration of most chloroallyl alcohols had decreased to levels below the detection limit (10 μM) (Table 1). Incubation with activated sludge gave slightly better conversion percentages compared to soil incubations. With both inocula, *trans*–2,3-dichloroallyl alcohol could still be detected after 10 days, showing a higher recalcitrance of this compound. The decrease in chloroallyl alcohol concentrations was due to biodegradation, since no decrease in concentration was observed in incubations with sodium azide added (data not shown).

Bacterial cultures known to degrade chlorinated aliphatic compounds were tested for their ability to grow on, or cometabolically convert, chloroallyl alcohols.

Table 1 Degradation of chloroallyl alcohols incubated with activated sludge and soil

Compound[a]	Chloroallyl alcohol (μM)			
	Activated sludge		Soil	
	$t=0$	$t=10^b$	$t=0$	$t=10^b$
Allyl alcohol	0.0	0.0	0.0	0.0
2-Chloroallyl alcohol	62.6	0.0	41.7	0.0
trans-3-Chloroallyl alcohol	33.3	0.0	47.0	0.0
cis-3-Chloroallyl alcohol	27.5	0.0	73.3	12.4
trans-2,3-Chloroallyl alcohol	31.5	10.3	76.1	18.6
cis-2,3-Chloroallyl alcohol	1.3	0.0	1.9	0.0
3,3-Dichloroallyl alcohol	47.2	0.0	60.2	6.9
Trichloroallyl alcohol	44.7	0.0	66.7	28.8

[a] Chloroallyl alcohols (100 μM) were added separately to different incubations except for *cis*- and *trans*-2,3-dichloroallyl alcohol, which were added as a mixture (75% and 25% respectively)
[b] Concentrations were determined after 10 days of incubation at 20° C in the dark

Growth was tested by replica plating on MMY/agar plates supplemented with chloroallyl alcohols and also in liquid medium containing 1 mM specific chloroallyl alcohol. Out of 16 pure cultures, including strains isolated on allyl alcohol, 2-chloroethanol, epichlorohydrin, 2,3-dichloropropanol and isoprene, only one strain was capable of growing on a chlorinated allyl alcohol. Growth on *trans*–3-chloroallyl alcohol was found with strain FG41 but this organism did not grow on other chloroallyl alcohols.

Oxidative cometabolic conversion of chloroallyl alcohols was tested by incubating cell suspensions of methane-grown cultures of *M. trichosporium* OB3b and *M. methanica* NCIB 11130. Both strains converted allyl alcohol, *trans*– and *cis*–3-chloroallyl alcohols, and 3,3-dichloroallyl alcohol, with complete removal of these compounds by *M. trichosporium* OB3b within 24 h (Table 2). Removal of a specific chloroallyl alcohol was accompanied by the formation of a chlorinated product, since no chloride production was observed. These products were identified with GC mass spectrometry (MS), revealing compounds with parent molecular ions at $m/z = 90$ (formed from *trans*– and *cis*–3-chloroallyl alcohol) and $m/z = 124$ (from 3,3-dichloroallyl alcohol), indicating the loss of two protons due to the oxidation of the chloroallyl alcohol to the chloroacrolein.

Cometabolic degradation of chloroallyl alcohols by cell suspensions of *X. autotrophicus* GJ10, which can grow on 2-chloroethanol and 1-chloro-3-propanol, was also restricted to β-chlorinated allyl alcohols. A more restricted substrate range was found with dichloromethane-grown cells of *Hyphomicrobium* GJ21, where only *trans*–3-chloroallyl alcohol was converted. No aldehydes were detected in incubations with these strains.

Isolation of organisms utilizing chloroallyl alcohols

Since no organisms were available that could carry out significant dechlorination, we attempted to isolate bacteria that could grow on chloroallyl alcohols. The occurrence of bacteria that are able to grow with chloroallyl alcohols as their sole carbon and energy source was investigated by repeatedly transferring samples from the initial incubations to fresh medium containing one specific chloroallyl alcohol. From the successful enrichments, eight different strains that could grow on one or more chloroallyl alcohols were isolated. Seven organisms (strains JD1 to JD6, and strain JD8) were isolated from incubations with exposed soil. Strain JD7 was isolated from the exposed activated sludge sample. Upon repeated transfers into fresh medium, no bacterial growth could be observed in enrichments started with two non-exposed inocula (activated sludge from a household sewage plant and harbor sludge).

The substrate range of the pure cultures was investigated by testing various compounds (Table 3). Growth was positive on ethanol, *n*–propanol, *n*–butanol, *n*–pen-

Table 2 Biodegradation of chloroallyl alcohols by pure cultures. *ND* not determined

Strain	Isolated on	Incubation time (h)	Remaining concentrations (mM)							
			Allyl alcohol	2-Chloroallyl alcohol	*trans*-3-Chloroallyl alcohol	*cis*-3-Chloroallyl alcohol	*trans*-2,3-Chloroallyl alcohol	*cis*-2,3-Chloroallyl alcohol	3,3-Dichloroallyl alcohol	Trichloroallyl alcohol	Cl^-
M. trichosporium OB3b[b]	Methane	24	<0.01	0.31	<0.01	<0.01	0.24	0.03	<0.01	0.48	<0.1
M. methanica 11130[b]	Methane	24	<0.01	0.33	0.05	0.02	0.23	0.03	0.18	0.44	<0.1
X. autotrophicus GJ10[a]	1,2-Dichloroethane	70	<0.01	0.34	0.02	<0.01	0.23	0.03	<0.01	0.44	ND
Hyphomicrobium GJ21[a]	Dichloromethane	70	0.37	0.29	<0.01	0.17	0.25	0.03	0.27	0.46	ND
Starting concentration			0.32	0.28	0.19	0.15	0.24	0.03	0.26	0.49	

[a] Late-exponential cultures, pregrown on the substrate that was used to isolate a particular organism, were supplemented with a particular chloroallyl alcohol (0.5 mM); *cis*- and *trans*-2,3-dichloroallyl alcohol were added as a mixture (respectively 75% and 25%)

[b] Late-exponential cultures, pregrown on methane (added in the gas phase, 15%, v/v) were supplemented with formate (20 mM) and a particular chloroallyl alcohol (0.5 mM); *cis*- and *trans*-2,3-dichloroallyl alcohol were added as a mixture (respectively 75% and 25%)

Table 3 Growth of pure cultures of *Pseudomonas* on (chlorinated) substrates. + growth visible within 1 week; − no growth; *x* growth substrate used for isolation

Substrate	Strains							
	JD6	JD8	JD4	JD7	JD5	JD1	JD2	JD3
Allyl alcohol	*x*	+	*x*	+	*x*	+	+	+
2-Chloroallyl alcohol	−	−	−	−	−	*x*	*x*	*x*
t-3-Chloroallyl alcohol	−	−	−	*x*	+	−	−	+
c-3-Chloroallyl alcohol	−	*x*	+	+	+	+	+	+
2,3-Dichloroallyl alcohol	−	−	−	−	−	−	+	+
3,3-Dichloroallyl alcohol	−	−	−	−	−	−	−	−
Trichloroallyl alcohol	−	−	−	−	−	−	−	−
2-Chloroethanol	−	−	−	−	−	−	−	+
1-Chloro-3-propanol	−	−	+	−	+	−	−	+
2-Chloro-1-propanol	−	−	−	−	−	+	+	+
2,3-Dichloropropanol	−	−	−	−	−	−	−	+
Chloroacetic acid	−	−	−	−	−	+	+	+
Dichloroacetic acid	−	−	−	−	−	+	+	+
Bromoacetic acid	−	−	−	−	−	+	+	+
2-Chloropropionic acid	−	−	−	−	+	+	+	+
3-Chloropropionic acid	−	+	+	+	+	−	−	+

tanol, *n*–hexanol, *n*–octanol, acetic acid, propionic acid, citric acid, glucose and allyl alcohol, but not on methanol, secondary alcohols, toluene, acetone, *cis*– and *trans*–3-chloroacrylic acid, 1-chloro-2-propanol, and acrolein. Differences between the isolates were mainly visible in the range of chlorinated compounds that could be used for growth. The ability of an organism to grow on allyl alcohol did not imply that chlorinated compounds, including chlorinated allyl alcohols, could be used as a growth substrate (e.g. strain JD6). Strain JD3, however, could use 13 different chlorinated compounds for growth, including 2,3- dichloroallyl alcohol, 2,3-dichloropropanol and several halogenated carboxylic acids. Weak growth on agar plates was observed on the mixtures of *cis*– and *trans*–2,3-dichloroallyl alcohol with strains JD2 and JD3. No growth was detected in liquid culture with these compounds. All organisms able to grow on 2-chloroallyl alcohol also utilized 2-haloalkanoic acids and 2-chloro-1-propanol.

The isolates were characterized with the Biolog identification system. All strains were catalase- and oxidase-positive, gram-negative rods. Mobility could sometimes be observed with strains JD5, JD6 and JD7, depending on cultural conditions. A good identification was obtained for strains JD1, JD2 and JD3 (*Pseudomonas fluorescens* subgroup B) and JD7 (*P. fluorescens*, subgroup not identified). The identification of strain JD2 as *P. fluorescens* was confirmed using fatty-acid profile analysis (Microbial Identification System Inc., Newark, Del). Strains JD4 and JD5 could be identified to the genus *Pseudomonas*). Strains JD6 and JD8 could not be identified with this test, morphological and physiological characteristics, however, suggest that these organisms also belong to the genus *Pseudomonas* (Palleroni 1984).

Since growth was found only with four or five out of the seven chloroallyl alcohols, we tested whether cometabolic degradation of the higher chlorinated analogs was possible. The eight organisms tested showed different capabilities of degrading chloroallyl alcohols cometabolically (Table 4). In general, *cis*–3-chloroallyl alcohol was easily converted by most organisms, while *trans*–2,3-dichloroallyl alcohol and trichloroallyl alcohol were persistent. The strains isolated on and incubated with 2-chloroallyl alcohol (JD1, JD2 and JD3) showed the best conversion of chloroallyl alcohols with degradation to below the detection limit (10 μM) in most incubations. The amount of chloride produced was low compared to the degree of substrate conversion, indicating formation of one or more chlorinated intermediates. In incubations of strains that had been isolated on and incubated with allyl alcohol (JD4, JD5

Table 4 Cometabolic conversion of chloroallyl alcohols by pure cultures of *Pseudomonas*. The growth substrate (in bold type) was added to a concentration of 2.5 mM, the other chloroallyl alcohols (2-chloroallyl alcohol to trichloroallyl alcohol) to a concentration of 0.25 mM each. 2,3-Dichloroallyl alcohol was added as a mixture of the *cis*- and *trans*-isomers to a concentration of 0.25 mM. The sterile control did not contain allyl alcohol.—Not added

Strain	Concentrations after 4 days (mM)									
	Allyl-alcohol	2-Chloro-allyl alcohol	*t*-3-Chloro-allyl alcohol	*c*-3-Chloro-allyl alcohol	*t*-2,3-Di-chloroallyl alcohol	*c*-2,3-Di-chloroallyl alcohol	3,3-Di-chloroallyl alcohol	Trichloro-allyl alcohol	Degradation[a] (%)	Dechlorination[b] (%)
JD1	—	**<0.01**	<0.01	<0.01	<0.01	<0.01	<0.01	0.14	88	27
JD2	—	**<0.01**	<0.01	<0.01	<0.01	0.01	<0.01	0.11	90	45
JD3	—	**<0.01**	<0.01	<0.01	0.07	<0.01	<0.01	<0.01	94	57
JD4	**<0.01**	0.10	0.06	<0.01	0.11	0.07	0.10	0.29	55	41
JD5	**<0.01**	<0.01	<0.01	<0.01	0.08	<0.01	<0.01	0.11	87	46
JD6	**<0.01**	0.04	<0.01	<0.01	0.07	0.07	0.06	0.29	68	73
JD7	—	0.08	**0.17**	<0.01	0.11	0.06	0.23	0.28	42	0
JD8	—	0.24	0.18	**1.22**	0.20	0.07	0.25	0.31	5	0
Sterile control	—	0.27	0.20	0.18	0.23	0.09	0.29	0.35	<5	<10

[a] Amount of chloroallyl alcohol converted (in addition to the growth substrate) relative to initial concentration
[b] Degree of dechlorination of degraded chloroallyl alcohols not added as growth substrate

Table 5 Cometabolic conversion of trichloroallyl alcohol by *Pseudomonas* strains

Strain	Carbon source	Concentration (mM)		Cell dry wt. (mg/ml)	$[Cl^-]$ (mM)	Degradation[b] (%)	Dechlorination[c] (%)
		Added[a]	Left				
JD2	2-Chloroallyl alcohol	2.5	<0.01	0.09	2.71	>95	>95
JD2	2-Chloroallyl alcohol	2.5	<0.01	0.08	3.40	82	56
	Trichloroallyl alcohol	0.5	0.09				
JD3	2-Chloroallyl alcohol	2.5	<0.01	0.08	2.68		
JD3	2-Chloroallyl alcohol	2.5	<0.01	0.08	3.55	96	60
	Trichloroallyl alcohol	0.5	0.02				
Sterile control	2-Chloroallyl alcohol	2.5	2.16	—	0.01	0	0
	Trichloroallyl alcohol	0.5	0.51				

[a] 2-Chloroallyl alcohol was added to a final concentration of 2.5 mM, trichloroallyl alcohol to a final concentration of 0.5 mM
[b] Amount converted trichloroallyl alcohol relative to initial concentration
[c] Chloride production relative to trichloroallyl alcohol conversion, corrected for 2-chloroallyl alcohol dechlorination

and JD6), some dechlorination of chloroallyl alcohols could be observed. Strains JD7 and JD8 did not utilize their growth substrate completely, and most chloroallyl alcohols were persistent in incubations with these strains. In esterified samples of incubations of strain JD7 with *trans*–3-chloroallyl alcohol, *trans*–3-chloroacrylic acid methylester was detected by GC-MS (parent molecular ion at $m/z = 120$).

The most persistent chloroallyl alcohol was trichloroallyl alcohol. Strains JD2 and JD3 appeared to be capable of cometabolic conversion of trichloroallyl alcohol during growth on 2-chloroallyl alcohol. The addition of 0.5 mM trichloroallyl alcohol did not have a negative effect on growth (Table 5). The chloride concentration in incubations with trichloroallyl alcohol present was higher than in the cultures containing only 2-chloroallyl alcohol, indicating that chloride was also liberated from trichloroallyl alcohol. The chloride concentration was lower than expected on the basis of the amount of trichloroallyl alcohol that was degraded, however, indicating partial conversion to a chlorinated product.

Cometabolic degradation of chloroallyl alcohols in continuous culture

Several attempts were made to obtain a stable continuous culture of strains JD2 and JD3 on 2-chloroallyl alcohol in a fermentor, but cultures repeatedly washed out at the start of the continuous feed. Inoculation of the fermentor with a mixture of 40-ml overnight cultures on 2-chloroallyl alcohol (strains JD1, JD2, JD3) and allyl alcohol (strain JD5) did result in a stable mixed culture that utilized chloroallyl alcohols in continuous culture. These strains were chosen because they were capable of degrading most chloroallyl alcohols either as a growth substrate or by cometabolism. Only when the system was operated under non-sterile conditions was a stable culture obtained, indicating that non-identified organisms stabilized the consortium. A cell recycle was used to increase the cell mass in the fermentor and prevent the cells from washing out. In this system, an adaptation period of 1 week was needed before stable conversion was observed. During this period, a biofilm was formed on the fermentor wall and the cell density in the medium decreased to 0.01 mg cell dry weight.ml^{-1}, upon which the cell recycle was switched off. 2-Chloroallyl alcohol was degraded in this continuous culture with 93% conversion of the substrate and a 98% dechlorination (Table 6, steady state 1). This implies that nearly complete mineralization of 2-chloroallyl alcohol was achieved, without the production of chlorinated byproducts.

After a stable culture had been obtained, other chlorinated allyl alcohols were sequentially added. First, *cis*– and *trans*–3-chloroallyl alcohol were added together with strains JD8 and JD7. After an adaptation period of 3 days, the mixed culture degraded both compounds to levels below the detection limit. Degradation was accompanied by the concomitant release of stoichiometric amounts of chloride, indicating complete mineralization of these compounds (steady state 2).

Addition of 3,3-dichloroallyl alcohol to the influent feed resulted in complete removal of this compound from the culture fluid without additional chloride production (steady state 3). This indicates that 3,3-dichloroallyl alcohol was converted to a chlorinated intermediate, without dechlorination taking place. Addition of *cis*– and *trans*–2,3-dichloroallyl alcohol to the influent did not influence the conversion of the other chloroallyl alcohols. Both compounds were converted, leading to the production of stoichiometric amounts of chloride and low concentrations of these compounds in the discharge (steady state 4, Fig. 1).

At this stage the composition of the microflora in the biofilm was examined. A sample of the biofilm was dispersed in buffer and substrate utilization of different bacteria in the biofilm was compared by replica plating on different media. It was found that colonies that could grow on *cis*–3-chloroallyl alcohol also showed growth on 2-chloroallyl alcohol, indicating the presence of bacteria similar to JD1, JD2 and JD3. Apparently,

Table 6 Biodegradation of chloroallyl alcohols in continuous culture. *Degr.* degradation, *Dechl.* dechlorination

Steady state number[a]	Dilution rate (h^{-1})	Allyl alcohol (substrates)	Concentration (mM)		Degr.[b] (%)	Dechl.[c] (%)	Cell dry wt.	$[Cl^-]$ (mM)
			In	Out				
1	0.10	2-Chloroallyl alcohol	2	0.14	93	98	0.03	1.82
2	0.05	2-Chloroallyl alcohol	2	0.09	97	91	0.02	2.65
		t-3-Chloroallyl alcohol	0.5	<0.01				
		c-3-Chloroallyl alcohol	0.5	<0.01				
3	0.05	2-Chloroallyl alcohol	2	0.02	99	58	<0.01	2.32
		t-3-Chloroallyl alcohol	0.5	<0.01				
		c-3-Chloroallyl alcohol	0.5	<0.01				
		3,3-Dichloroallyl alcohol	0.5	<0.01				
4	0.05	2-Chloroallyl alcohol	2	0.02	98	74	<0.01	4.35
		t-3-Chloroallyl alcohol	0.5	<0.01				
		c-3-Chloroallyl alcohol	0.5	<0.01				
		t-2,3-Dichloroallyl alcohol[d]	0.5	0.06				
		c-2,3-Dichloroallyl alcohol	0.5	<0.01				
		3,3-Dichloroallyl alcohol	0.5	<0.01				
5	0.05	2-Chloroallyl alcohol	2	0.02	97	78	<0.01	4.88
		t-3-Chloroallyl alcohol	0.5	<0.01				
		c-3-Chloroallyl alcohol	0.5	<0.01				
		t-2,3-Dichloroallyl alcohol	0.5	0.09				
		c-2,3-Dichloroallyl alcohol	0.5	<0.01				
		3,3-Dichloroallyl alcohol	0.5	<0.01				
		Trichloroallyl alcohol	0.5	0.01				
6	0.03	2-Chloroallyl alcohol	5	0.11	98	94	<0.01	4.61
7	0.03	2-Chloroallyl alcohol	5	1.41	71	76	<0.01	3.45
		Trichloroallyl alcohol	0.5	0.19				
8	0.05	2-Chloroallyl alcohol	5	0.04	99	80	<0.01	5.14
		Trichloroallyl alcohol	0.5	<0.01				
9	0.05	2-Chloroallyl alcohol	5	0.05	99	71	<0.01	6.57
		t-3-Chloroallyl alcohol	0.5	<0.01				
		c-3-Chloroallyl alcohol	0.5	<0.01				
		t-2,3-Dichloroallyl alcohol	0.25	0.05				
		c-2,3-Dichloroallyl alcohol	0.25	<0.01				
		3,3-Dichloroallyl alcohol	0.5	<0.01				
		Trichloroallyl alcohol	0.5	0.01				
10	0.05	2-Chloroallyl alcohol	—	—	64	20	<0.01	0.52
		t-3-Chloroallyl alcohol	0.5	<0.01				
		c-3-Chloroallyl alcohol	0.5	0.19				
		t-2,3-Dichloroallyl alcohol	0.25	0.22				
		c-2,3-Dichloroallyl alcohol	0.25	0.02				
		3,3-Dichloroallyl alcohol	0.5	0.14				
		Trichloroallyl alcohol	0.5	0.33				

[a] Steady states were measured after at least five volume changes, and are a result of two to five independent samples taken during 1 or more days

[b] Amount of substrate converted relative to initial concentration

[c] Cloride production relative to substrate conversion

[d] *trans-* and *cis*-2,3-dichloroallyl alcohol were added as a mixture (1:1)

addition of strains JD7 and JD8 to the fermentor had not resulted in incorporation of these strains as dominant organisms in the biofilm. Colonies that could grow on 2,3-dichloropropanol also showed growth on 2-chloroallyl alcohol, and therefore resemble strain JD3. However, some of these colonies failed to grow on *cis*– or *trans*–3-chloroallyl alcohol, and thus have a pattern of chloroallyl alcohol utilization different from that of the isolates that were used to inoculate the fermentor. Finally, not all colonies that grew on 2-chloroallyl alcohol could grow on 2,3-dichloropropanol, indicating the presence of strains similar to JD1 and JD2.

The continuous-culture degradation experiment was continued with addition of trichloroallyl alcohol to the influent. The compound was degraded with nearly complete conversion, leading to some chloride production (steady state 5) but less than expected on the basis of the amount of trichloroallyl alcohol degraded. Since many different chlorinated substrates are present in the influent at this stage, it is impossible to distinguish between chloride release from the different components in the influent, however. Apparently, chlorinated intermediates are formed during the conversion of the chloroallyl alcohols. The omission of yeast extract from the medium did not affect fermentor performance, indicating that a stable biofilm was formed that was able to grow on and cometabolically convert chloroallyl alcohols without any additional carbon source (data not shown).

A second fermentor was constructed and inoculated in an identical manner to that in the previous experiment. 2-Chlororallyl alcohol served as the growth sub-

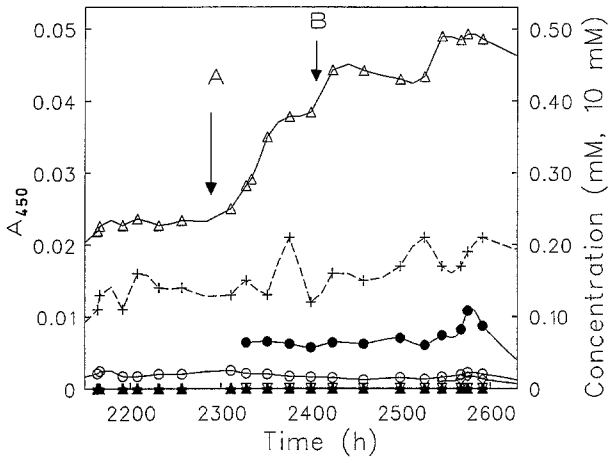

Fig. 1 Biodegradation of chloroallyl alcohols in continuous culture. The temperature was 30° C, the pH was kept constant at 7.0 and the dilution rate was 0.05 h^{-1}. *A* addition of *trans*– and *cis*–2,3-dichloroallyl alcohol (0.5 mM each); *B* addition of 0.5 mM trichloroallyl alcohol, + cell density (A_{450}), △ chloride, ▲ 2-chloroallyl alcohol, + *trans*–3-chloroallyl alcohol, ○ *cis*–3-chloroallyl alcohol; ● *trans*–2,3-dichloroallyl alcohol, ▽ *cis*–2,3-dichloroallyl alcohol, ♦ trichloroallyl alcohol. Chloride concentrations are expressed in 10 mM, other concentrations in mM

strate, and the stability of the biofilm was tested in a series of experiments (Table 6). The effect of the addition of trichloroallyl alcohol was tested in the fermentor 8 days after the biofilm formed and a stable fermentor performance was found (Table 6, steady state 6). This had a temporary negative effect on the biodegradation of 2-chloroallyl alcohol, and degradation of trichloroallyl alcohol was incomplete (Table 6, steady state 7). After an adaptation period of 7 days 99% conversion of both compounds was reached (steady state 8). Apparently, the composition of the biofilm had changed during the course of the experiment towards strains that were not inhibited by trichloroallyl alcohol and were essential for the degradation of this compound.

The addition of the other chloroallyl alcohols to the fermentor did not have any effect on 2-chloroallyl alcohol degradation, indicating that a stable biofilm was formed. After five volume changes, nearly complete degradation (99%) of all added chloroallyl alcohols was found, leading to a 71% release of the organically bound chloride (steady state 9). These data are in good agreement with the results obtained with the other fermentor, indicating that formation of a biofilm and fermentor performance are reproducible. At this stage, a sample of the biofilm was dispersed in buffer and plated on non-selective medium and on MMY, supplemented with 2-chloroallyl alcohol. It was found that the primary consumers (growing on 2-chloroallyl alcohol) comprised 79% of the total colony-forming units in the biofilm.

Omitting 2-chloroallyl alcohol from the influent resulted in loss of chloroallyl-alcohol-degrading potential of the biofilm (steady state 10). In particular, the degradation of *trans*–2,3-dichloroallyl alcohol and trichloroallyl alcohol was blocked, indicating that the degradation of these compounds depended on cometabolism. Dechlorination of the other compounds was also negatively influenced. No steady state was reached, and the experiment was stopped after eleven volume changes.

We then tested whether the addition of the cultures of chloroallyl-alcohol-utilizing strains was essential for the start-up of a continuous culture. A fermentor was inoculated with 100 ml activated sludge, freshly collected from a household sewage treatment plant, and 100 μM 2-chloroallyl alcohol was added. After 24 h, the medium pump was activated, and a mixture of chloroallyl alcohols (2 mM 2-chloroallyl alcohol, 0.5 mM *trans*– and *cis*–3-chloroallyl alcohol, 0.25 mM *trans*– and *cis*–2,3-dichloroallyl alcohol, 0.5 mM 3,3-dichloroallyl alcohol and 0.5 mM trichloroallyl alcohol) was continuously supplied. The dilution rate was set at 0.05 h^{-1}, and the cell recycle was activated. After 7 days, the chloroallyl alcohol concentrations in the fermentor were the same as in the influent, and no chloride production could be observed. Thus, continuous degradation of chloroallyl alcohols was not obtained with the microflora present in the activated sludge. Start-up of a fermentor with a defined coculture of four different chloroallyl-alcohol-degrading strains, however, repeatedly led to the formation of a stable continuous culture.

Discussion

Biodegradation experiments in which chloroallyl alcohols were incubated with activated sludge, harbor sludge, soil samples, or various pure cultures capable of degrading other chlorinated aliphatic compounds and allyl alcohol indicated that the capacity to utilize chloroallyl alcohols as a growth substrate is not widespread among bacteria. Especially the presence of a chlorine substituent on the α-carbon hindered transformation by most cultures. This is not the case with *Pseudomonas fluorescens* JD2, where an alcohol dehydrogenase is present that has good activity with 2-chloroallyl alcohol, which may explain the recalcitrance of chloroallyl alcohols in the absence of strains similar to JD2 (van der Waarde et al. 1993).

Methanotrophic bacteria have been found to convert a large series of chlorinated hydrocarbons cometabolically by oxygenation (Oldenhuis et al. 1989) but *trans*–3-chloro-, *cis*–3-chloro-, and 3,3-dichloroallyl alcohol were converted to the corresponding aldehydes rather than to epoxides. No differences were observed between *M. trichosporium* OB3b, expressing the soluble-type methane monooxygenase, or strain *M. methanica* 11130, expressing the particulate-type monooxygenase. Apparently, the alcohol function prevents monooxygenation, since 1,3-dichloropropylene is converted by the soluble methane monooxygenase (Olden-

huis et al. 1989). Only the β-chloroallyl alcohols were converted by an alcohol dehydrogenase to the corresponding chloroacroleins without further conversion or dechlorination.

After enrichment with (chloro)allyl alcohols as growth substrate, eight different pure cultures could be isolated from a site with a history of chloroallyl alcohol contamination. No strains could be isolated from two non-exposed environmental samples. The strains appeared to be strongly related, both on the basis of their morphological appearance on agar plates and in liquid medium culture, and on the basis of their catabolic potential. Apart from different behavior in the Biolog test, clear differences between the strains only existed in the utilization pattern of chlorinated compounds. It is remarkable that the ability to grow on 2-chloroallyl alcohol and α-chlorinated carboxylic acids as well as on 2-chloropropanol seemed to be coupled. It is not known whether the latter compounds are intermediates in a minor metabolic pathway of 2-chloroallyl alcohol, which was previously found to be degraded via 2-chloroacrylic acid (van der Waarde et al. 1993). It has also been demonstrated that *trans*– and *cis*–3-chloroallyl alcohol are degraded via the corresponding chloroacrylic acids by two unidentified *Pseudomonas* sp. (van der Waarde et al. 1993).

Several chloroallyl alcohols that did not serve as a growth substrate could be cometabolically transformed and dechlorinated by some of the isolates obtained, including an organism not capable of utilizing any chloroallyl alcohol. This is an indication that dechlorination of chloroallyl alcohols is not carried out by a specific dehalogenase, but is a result of aspecific reactions. A different situation is found with the dechlorination of *trans*– and *cis*–3-chloroacrylic acid, which are converted by specific dehalogenases (Hartmans et al. 1991; van Hylckama Vlieg and Janssen 1992). The best cometabolic degradation of chloroallyl alcohols was found with strains JD2 and JD3, isolated on 2-chloroallyl alcohol. Cometabolic dechlorination of the highest chlorinated and most recalcitrant analog, trichloroallyl alcohol, by these strains was possible during growth on 2-chloroallyl alcohol, leading to 60% dechlorination of the trichloro compound.

Until now, no pure culture has successfully been grown in continuous culture under aseptic conditions. Only the use of a mixed culture of chloroallyl-alcohol-degrading organisms (strains JD5, JD1, JD2, and JD3) has led to the formation of biofilm culture that could be maintained under non-sterile conditions for continuous breakdown of chloroallyl alcohols. The system was very stable and has been in operation for 4 months with nearly 100% degradation and dechlorination of 2-chloroallyl alcohol, *cis*– and *trans*–3-chloroallyl alcohol, and *cis*– and *trans*–2,3-dichloroallyl alcohol. 3,3-Dichloroallyl alcohol was also degraded to levels below the detection limit, but did not seem to be dechlorinated. Probably, 3,3-dichloroacrylic acid accumulated in this case, in agreement with the complete conversion of 3,3-dichloroallyl alcohol to 3,3-dichloroacrylic acid by resting cells of strain JD2 (van der Waarde et al. 1993). Trichloroallyl alcohol was also degraded in continuous culture. The biofilm needed an adaptation period before conversion was stable after which 99% conversion was achieved. Chloride was released, but no complete mineralization was found. A similar phenomenon was observed with cells of strain JD2 incubated with trichloroallyl alcohol (van der Waarde et al. 1993). Apparently, the metabolism of trichloroallyl alcohol leads to chlorinated intermediates that accumulate in the medium.

The results indicate that degradation of most chloroallyl alcohols in continuous culture is dependent on cometabolism. For example, oxidation of *trans*–2,3-dichloroallyl alcohol was only possible during growth of the mixed culture on 2-chloroallyl alcohol. Probably, the enzymes involved in 2-chloroallyl alcohol degradation are not very specific and can convert a range of substrates, as has been shown for the alcohol dehydrogenase of strain JD2 (van der Waarde et al. 1993).

The establishment of a stable system for continuous chloroallyl alcohol degradation always correlated with biofilm formation, stressing the importance of intercellular interactions in the biodegradation process. Excellent removal efficiencies were obtained in the absence of additional organic carbon sources such as yeast extract, as opposed to, for example, treatment of waste water contaminated with glyphosate (Hallas et al. 1992). Most likely, the biofilm that is responsible for these conversions is composed of a mixture of chloroallyl-alcohol-degrading strains and prevents the organisms from exposure to toxic levels of the substrates and their degradation products. The combination of activities present in the mixed culture may also increase the catabolic potential over that of pure cultures. Bacteria other than the cultures added must also play an essential role in biofilm formation since it was not possible to obtain a stable continuous culture with a mixture of chloroallyl-alcohol-degrading strains under sterile conditions. Indeed the biofilm was composed of different types of bacteria. About 80% of the bacteria could actually grow on the primary substrate, 2-chloroallyl alcohol. This group of primary consumers included more than one strain. Similar observations were made by Bitzi et al. (1991), who found that enrichment in a continuous culture for bacteria growing on methanol, methylene chloride, acetone and isopropanol resulted in the formation of an essentially binary (99%) culture. The satellite strains found in their research did not affect removal efficiencies. Another interesting observation that we made is that, in the biofilm, chloroallyl-alcohol-degrading strains were present with a catabolic potential that was not present in the strains that were used to inoculate the fermentor, which could be the result of genetic changes of the bacteria used for inoculation.

It is clear from these results that all chloroallyl alcohols are biodegradable, and that mineralization requires the presence of both specialized strains and a suitable growth substrate. This makes the prospect of a

dedicated biological purification system for the treatment of chloroallyl-alcohol-containing waste water feasible. Extensive dechlorination of even highly chlorinated analogs seems possible. Further research into the aerobic cometabolic processes and the performance of a fixed film reactor system should enable the development of an efficient biological treatment system for the purification of waste water contaminated with chlorinated allyl alcohols.

References

Bergman J, Sanik J (1957) Determination of trace amounts of chlorine in naphtha. Anal Chem 29:241–243

Bitzi U, Egli T, Hamer G (1991) The biodegradation of mixtures of organic solvents by mixed and monocultures of bacteria. Biotechnol Bioeng 37:1037–1042

Ensley BD (1991) Biochemical diversity of trichloroethylene metabolism. Annu Rev Microbiol 45:283–299

Hallas LE, Adams WJ, Heitkamp M (1992) Glyphosate degradation by immobilized bacteria: field studies with industrial waste water effluent. Appl Environ Microbiol 58:1215–1219

Hartmans S, Jansen MW, Werf MJ van der, Bont JAM de (1991) Bacterial metabolism of 3-chloroacrylic acid. J Gen Microbiol 137:2025–2032

Hylckama Vlieg JET van, Janssen DB (1992) Bacterial degradation of 3-chloroacrylic acid and the characterization of *cis*- and *trans*-specific dehalogenases. Biodegradation 2:139–150

Janssen DJ, Scheper A, Witholt B (1984) Biodegradation of 2-chloroethanol and 1,2-dichloroethane by pure bacterial cultures. Progress Ind Microbiol 20:169–178

Kohler H-PE, Kohler-Staub D, Focht DD (1988) Cometabolism of polychlorinated biphenyls: enhanced transformation of Aroclor 1254 by growing bacterial cells. Appl Environ Microbiol 54:1940–1945

Meier JR, Ringhand HP, Coleman WE, Munch JW, Streicher RP, Kaylor WH, Schenck KM (1985) Identification of mutagenic compounds formed during chlorination of humic acid. Mutat Res 157:111–122

Motosugi K, Soda K (1983) Microbial degradation of synthetic organochlorine compounds. Experientia 39:1214–1220

Oldenhuis R, Vink RLJM, Janssen DB, and Witholt B (1989) Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl Environ Microbiol 55:2819–2826

Palleroni NJ (1984) Genus *Pseudomonas*. In: Krieg NR (ed) *Bergeys's manual of systematic bacteriology*, vol 1. Williams & Wilkins, Baltimore, pp 141–199

Rosen JD, Segall Y, Casida JE (1980) Mutagenic potency of haloacroleins and related compounds. Mutat Res 78:113–119

Tachibana S, Santodonato J, Dence CW (1989) Reactions of selected nucleophiles with 2-chloropropenal, a mutagenic constituent of softwood kraft chlorination effluent. Environ Tox Chem 8:1133–1139

Thurnheer T, Cook AM, Leisinger T (1988) Co-culture of defined bacteria to degrade seven sulfonated aromatic compounds: efficiency, rates and phenotypic variations. Appl Microbiol Biotechnol 29:605–609

Waarde JJ van der, Kok R, Janssen DB (1993) Microbial degradation of 2-chloroallyl alcohol. Appl Environ Microbiol 59:528–535